

Structure, Mechanism, and Regulation of Mammalian Adenylyl Cyclase*

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The discovery of 3',5'-cyclic adenosine monophosphate (cAMP) in the late 1950s by Sutherland and co-workers was the pivotal event that led to our current paradigm of hormone signaling through second messengers. Despite the subsequent discovery of many other second messengers, cAMP has never left center stage. The adenylyl cyclases are the family of enzymes that synthesize cAMP (1–5).

Breakthroughs in determining the first structures of the mammalian adenylyl cyclase catalytic core (6, 7) provide a new context for understanding the action of many regulators, both physiological and pharmacological: free metal ions, P-site inhibitors, forskolin, G-proteins, Ca^{2+} /calmodulin, and protein phosphorylation. Understanding the catalytic mechanism of an enzyme is a prerequisite to understanding its regulation. Here I will describe the essentials of catalysis and then consider how these elements are controlled by each of the major regulators.

Structure of Adenylyl Cyclase

The nine cloned isoforms of mammalian adenylyl cyclase share a primary structure consisting of two transmembrane regions, M_1 and M_2 , and two cytoplasmic regions, C_1 and C_2 (8) (Fig. 1). The transmembrane regions each contain six predicted membrane-spanning helices. The function of M_1 and M_2 , aside from membrane localization, is unknown despite their topological analogy to transporters. The C_1 and C_2 regions are subdivided into C_{1a} and C_{1b} ; and C_{2a} and C_{2b} . The C_{1a} and C_{2a} are well conserved, homologous to each other, and contain all of the catalytic apparatus (9). C_{1a} and C_{2a} domains heterodimerize with each other in solution (10, 11). These domains can also form homodimers. Domains derived from different isoforms can form chimeric heterodimers. The C_{1b} region is large (~15 kDa), variable, and contains several regulatory sites. The C_{2b} is vanishingly short in some isoforms and lacks identified functions; hence C_2 and C_{2a} are sometimes referred to interchangeably.

The structure of the type II adenylyl cyclase C_2 region revealed a homodimer with two C_2 monomers in a wreath-like arrangement (6). A deep ventral groove runs between the two in the center of the wreath. Two forskolin molecules bind to this groove in the homodimer. The monomer is built around a large β sheet that folds back onto itself on the "inside" facing the dimer interface. The "outside" is α -helical. A ~80-amino acid substructure within the monomer is similar to the palm domains of the DNA polymerase I and reverse transcriptase families (12, 13).

The type V C_{1a} region and type II C_2 region arrange themselves in a heterodimeric wreath that is nearly identical in

overall structure to the C_2 homodimer, with some critical differences in detail (7). The active site is at one end of the ventral groove. The single forskolin binding site, as anticipated by equilibrium binding (14), is at the other. The active site is formed at the interface by residues contributed by both C_{1a} and C_2 . Because the active site is shared between the two domains, association of two catalytic domains in the proper orientation is an absolute prerequisite of catalytic activity. The activity of mammalian adenylyl cyclases depends on the heterologous association of C_{1a} and C_2 . This is not the case for many other related cyclases (15, 16). Mammalian membrane guanylyl cyclases and many microbial homologues of mammalian adenylyl cyclases are active as homodimers. The mammalian C_2 homodimer has measurable activity (9, 17, 18), although reduced by many orders of magnitude because of the loss of two catalytic Asp residues relative to the heterodimer.

Nucleotide Binding Site and Specificity

The ATP binding site has been revealed by the structure of the P-site inhibitor complex (7), molecular modeling (15), and mutagenic analysis (15, 19–22). Lys-923¹ and Asp-1000 from C_2 interact directly with the N-1 and N-6 of the adenine ring. Gln-417 of C_1 plays a supporting role by orienting the Lys. Mutation of these three residues destroys the ATP *versus* GTP nucleotide specificity of adenylyl cyclase, although it does not convert it into a guanylyl cyclase (22). This is because of a main chain carbonyl that hydrogen bonds to the adenine N-6 and disfavors guanine. Guanylyl cyclases can be converted completely into adenylyl cyclases by mutating their guanine binding Glu and Cys to their adenylyl cyclase counterparts, Lys and Asp (21, 22).

The ATP binding site is rounded out by hydrophobic residues that pack against the purine ring and by charged interactions with phosphate groups. Hydrophobic contacts are contributed mainly by C_2 . Charged interactions are formed by Arg (C_1) and Lys (C_2). The Lys is part of a flexible lid over the active site that is capable of undergoing an order-disorder transition (6, 7).

Mg^{2+} Binding Site

The Mg^{2+} binding site consists of two mutationally sensitive Asp residues (15, 19). The P-site inhibitor complex shows a single Mg^{2+} ion interacting with both phosphate moieties of pyrophosphate (7). There is abundant kinetic evidence for a two-ion mechanism (23, 24). One ion acts kinetically as free Mg^{2+} whereas the other binds as a complex with ATP. An analogous situation holds for the DNA polymerase family. The polymerases carry out the intermolecular attack of a primer 3'-hydroxyl on the α -phosphate of a deoxynucleotide rather than the intramolecular attack of a nucleotide 3'-hydroxyl on its own α -phosphate.

Two Mg^{2+} ions bind to DNA polymerase-primer-template-nucleotide complexes (25–28), providing a model for the coordination of two ions in the adenylyl cyclase active site. The polymerase "B" metal ion corresponds to the observed ion in the adenylyl cyclase complex. It binds all three nucleotide phosphates in a tridentate arrangement (25, 27). These tight interactions leave little doubt that this is the ion that acts kinetically as an ATP complex. The polymerase "A" metal ion is less

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¹ Unless the isoform is explicitly stated, residue numbering is for type I.

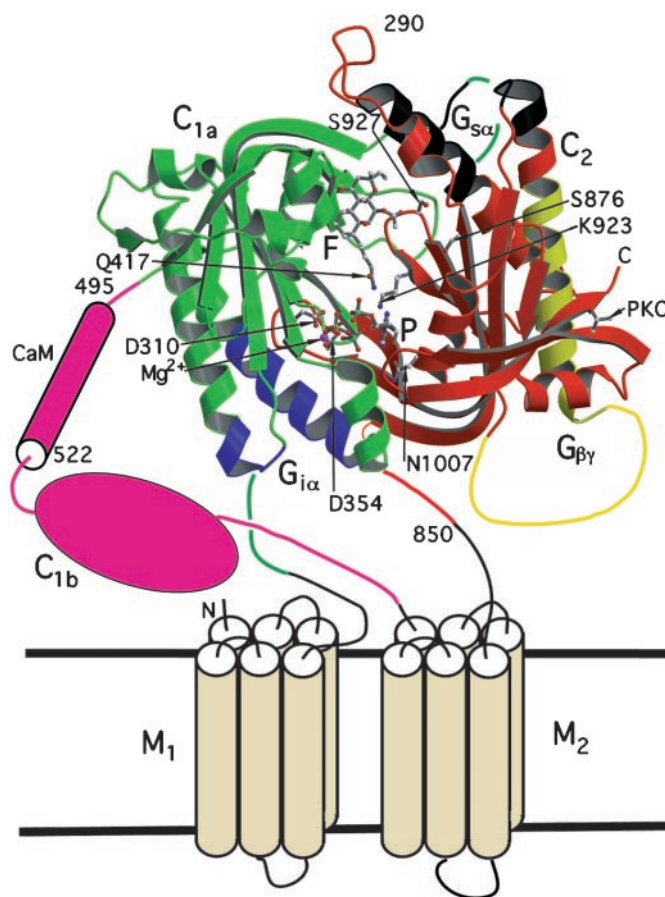


FIG. 1. **Structure of adenylyl cyclase.** Numbering is for type I. The crystal structure is shown for the type V C_1 (green)/type II C_2 (red) heterodimer bound to forskolin, adenosine 2'-deoxy-3'-monophosphate, pyrophosphate, and one Mg^{2+} (G_{sa} is omitted). Membrane (cream) and C_{1b} (magenta) regions are drawn. Binding sites for G_{sa} , $G_{i\alpha}$ (types V and VI), and $G_{\beta\gamma}$ (type II) are black, blue, and yellow, respectively. The Ca^{2+} /calmodulin binding helix shown is for type I, and the protein kinase C phosphorylation site shown is for type II. Atom colors are: carbon, light gray; nitrogen, blue; oxygen, red; phosphorus, green; and magnesium, purple. The orientation of the catalytic core relative to the membrane is arbitrary in this view.

tightly bound as judged by higher temperature factors and fewer interactions with protein and nucleotide (25). It interacts with the 3'-hydroxyl of the primer and the nucleotide α -phosphate. Both metal ions are coordinated by both Asps. This coordination geometry fits the adenylyl cyclase structure consistent with known stereochemistry (29).

Mechanism of Cyclic AMP Formation

Cyclic AMP formation requires the deprotonation and activation of the ATP 3'-hydroxyl for nucleophilic attack; stabilization of the transition state at the α -phosphate; and stabilization of increased negative charge on the leaving group, pyrophosphate. Metal ion A activates the 3'-hydroxyl, and both metal ions share in transition state stabilization. Asn-1007, Arg-1011, and Lys-1047 approach the phosphate moieties (Fig. 2). Their modeled interactions with the non-bridging α -phosphate oxygens have poor geometry. It seems likely that at least one of these residues stabilizes the leaving group. Their precise positions in the ATP complex and, therefore, their precise roles in catalysis have yet to be determined. The fate of the proton on the 3'-hydroxyl is unknown. It has been suggested that Asp-354 in adenylyl cyclase or its counterpart in DNA polymerase could act as a general base in these reactions (15, 26). Substrate-assisted catalysis is the other leading possibility suggested for base catalysis (7, 20).

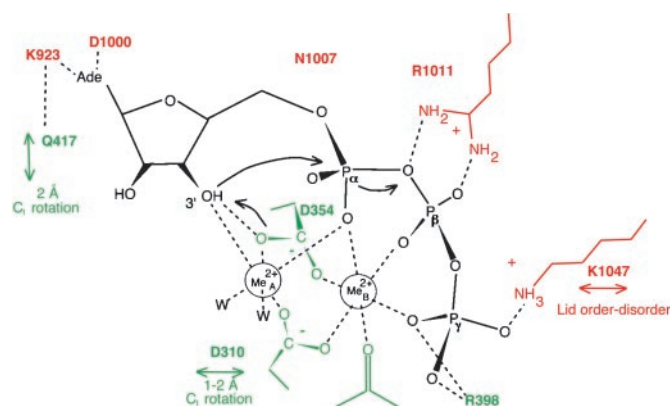


FIG. 2. **Model for the mechanism of adenylyl cyclase.** Metal coordination by phosphates and Asp is derived from the T7 DNA polymerase structure (25), and the mechanism is adapted from that of Steitz (28). C_1 residues are green, and C_2 residues are red. Numbering is for type I. Interactions regulated by conformational changes are marked. Asp-310 moves about 1 Å between the two conformations, and Asp-354 and Gln-417 move about 2 Å. A hypothetical hydrogen bond between the putative base Asp-354 and the ATP 3'-hydroxyl is shown. Arg-1011 probably interacts with the α - β bridging oxygen of ATP, but its other interactions are less certain.

A Conformational Change That Controls Domain Orientation and Active Site Structure

The determinants of both nucleotide binding and catalysis are shared between C_1 and C_2 . This insight is central to understanding regulation of adenylyl cyclase catalytic activity. It means that any factor that alters the relative orientation of the C_1 and C_2 domains can alter the structure of the active site and thereby alter substrate affinity, catalytic velocity, or both.

The structure of the catalytic core is known in two conformations: that of the forskolin-bound homodimer (6) and that of the forskolin and $G_{sa}\alpha^2$ -bound heterodimer (7). The two structures differ by a 7° rotation of the heterodimer C_1 domain relative to the correspondent C_2 in the homodimer. The domain rotation brings key catalytic elements from the two domains about 2 Å closer to each other. The structural differences between the two might be caused by differences in interface residues in the two different dimers, by occupancy of one *versus* two forskolin molecules, or, most probably, by the binding of $G_{sa}\alpha$. Despite some uncertainties about which of these factors are driving the observed structural change, there is no doubt that the two boughs of the adenylyl cyclase wreath are capable of moving into more or less active conformations.

Regulation by Free Metal Ions

Mammalian adenylyl cyclases are strongly activated by Mn^{2+} and inhibited by millimolar concentrations of free Ca^{2+} . These effects are unlikely to have any physiological meaning or to reflect distinct binding sites for these ions. Many otherwise unrelated Mg^{2+} -dependent enzymes can be activated by replacing Mg^{2+} with Mn^{2+} , probably because the latter is nearly the same size but is a stronger Lewis acid. At high concentrations free Ca^{2+} binds competitively to Mg^{2+} sites on enzymes but fails to replace it catalytically. A high affinity and possibly physiological inhibition of type V and VI adenylyl cyclase by free Ca^{2+} has been reported (30), and the possibility of a high affinity binding site in the C_{1b} domain of these isoforms has been raised (31).

² The abbreviations used are: $G_{sa}\alpha$, stimulatory G-protein α subunit; $G_{i\alpha}$, inhibitory G-protein α subunit; $G_{\beta\gamma}$, G-protein $\beta\gamma$ subunit; CaM kinase, Ca^{2+} /calmodulin-dependent protein kinase.

Regulation by P-site Inhibitors

P-site inhibitors are a class of nucleoside inhibitors of adenylyl cyclase so-called because they all contain a purine ring (32). The most potent lack a 2'-hydroxyl and are polyphosphorylated at the 3'-position (32–34). P-site inhibition is potentiated when adenylyl cyclase is activated. P-site inhibition is hypersensitive to certain mutations that slightly reduce enzyme activity. P-site inhibitors are non- or uncompetitive with respect to the forward reaction but compete with the product cyclic AMP in the reverse reaction (35). P-site inhibitors are effective against engineered ATP-specific guanylyl cyclases that are in all other ways regulated by different mechanisms than the adenylyl cyclases (22). P-site inhibitors bind to the active site primarily through conserved residues. Different adenylyl cyclases do show some differences in P-site inhibition, and a physiological role for this type of regulation has been postulated (36).

Regulation by Forskolin

Forskolin is a hydrophobic activator of all the mammalian adenylyl cyclases except type IX. It is an extremely powerful activator of some of the synthetic soluble adenylyl cyclase systems, increasing activity by up to 10^3 , although other forms of soluble adenylyl cyclase barely respond. Forskolin binds to the catalytic core at the opposite end of the same ventral cleft that contains the active site (7, 15). It activates the enzyme by gluing together the two domains in the core using a combination of hydrophobic and hydrogen bonding interactions that are distributed equally between the two domains (6). Type IX adenylyl cyclase is non-responsive to forskolin because of a Ser \rightarrow Ala and a Leu \rightarrow Tyr change in the binding pocket. When these changes are reversed by site-directed mutagenesis, the resulting type IX mutant can be activated by forskolin as well as other adenylyl cyclases (37).

The forskolin binding pocket is a narrow hydrophobic crevice that almost completely buries the forskolin molecule once bound. The pocket residues are absolutely conserved in types I–VIII and differ only subtly in type IX. The presence of a hydrophobic crevice in a protein is highly destabilizing in the absence of bound ligand. It seems improbable that such a destabilizing feature would be so highly conserved if it had no function. This paradox led us to revive the idea that there exists an endogenous forskolin-like small molecule activator of adenylyl cyclase.

Regulation by G-protein Subunits

All mammalian adenylyl cyclases are potently and physiologically activated by the GTP-bound G-protein α -subunit $G_s\alpha$. This activation is synergistic, not competitive, with respect to forskolin. GTP- $G_s\alpha$ binds to a crevice on the outside of the wreath formed by $\alpha 2'$ and $\alpha 3'$ of C_2 and by the N-terminal portion of C_1 (7, 38, 39). GTP- $G_s\alpha$ is capable of gluing together C_1 and C_2 as does forskolin, but mutational analysis suggests this cannot be its only function. If the C_1 contact is abolished, activation can be partially rescued when forskolin is used to dimerize C_1 and C_2 . Therefore there must be a non-glue role for GTP- $G_s\alpha$ (38). This role is probably to induce a conformational change that allosterically stimulates catalysis. The 7° rotation of C_1 , which moves the catalytic residues into their proper positions, is probably the result of a torque applied by $G_s\alpha$ as it “pushes” the C_1 away from its binding site (7).

$G_i\alpha$ selectively inhibits adenylyl cyclase types V and VI. Symmetry and sequence homology arguments led to the suggestion that $G_i\alpha$ binds to the adenylyl cyclase catalytic core on a groove pseudosymmetrically related to the $G_s\alpha$ binding groove (7, 38). Mutational analysis confirmed that the groove formed by $\alpha 2$ and $\alpha 3$ of C_1 is the primary site for binding of $G_i\alpha$

to type V (40). The inhibitory mechanism postulates a rotation of the C_1 in the opposite sense as that induced by $G_s\alpha$.

$G\beta\gamma$ subunits conditionally regulate several adenylyl cyclases. Type II adenylyl cyclase is activated by $G\beta\gamma$ when $G_s\alpha$ is bound. At least part of the $G\beta\gamma$ binding site of type II has been located using peptide competition studies (41). The site spans a flexible loop between $\beta 3'$ and $\alpha 3'$ and the first two-thirds of $\alpha 3'$ (6). The $G\beta\gamma$ site is adjacent to, but does not overlap, the $G_s\alpha$ site, consistent with conditional activation.

G-protein interactions with non-catalytic regions of adenylyl cyclase seem likely. The $\alpha 4$ – $\beta 6$ region of $G_s\alpha$ was predicted to interact with adenylyl cyclase based on mutagenic analysis (42, 43), but no such contact with the catalytic domain was seen in the crystal structure. $G\beta\gamma$ regulation of the soluble adenylyl cyclase model has not been established, even though the known binding site is located within the type II C_2 domain. C_{1b} (44), M_1 , or M_2 might be involved in either of these processes.

Regulation by Ca^{2+} /Calmodulin

Ca^{2+} /calmodulin activates type I adenylyl cyclase by binding to a putative helical region on the C_{1b} (45, 46). The precise activation mechanism is unknown. If other Ca^{2+} /calmodulin-activated enzymes are a precedent, it is likely that Ca^{2+} /calmodulin binding will disrupt an autoinhibitory interaction between the C_{1a} / C_2 catalytic core and sequences within the C_{1b} .

Regulation by Protein Phosphorylation

Protein kinase C activates type II adenylyl cyclase by phosphorylating it on Thr-1057 (47). This site is within a region known to be required for protein kinase C activation (48). This Thr is at the edge of the “lid,” a flexible region that is disordered in the homodimer structure but folds over the top of the active site in the heterodimer-P-site complex. Phosphorylation might enhance the ability of the lid to adopt the correct conformation. CaM kinase II inhibits type III adenylyl cyclase by phosphorylating it at Ser-1076 (49). This Ser is at the outer lip of the active site, hence its phosphorylation could directly interfere with catalysis. Protein kinase A phosphorylates Ser-674 in the C_{1b} of type VI (50) and appears to regulate a low affinity secondary binding site for $G_s\alpha$. CaM kinase IV phosphorylates type I adenylyl cyclase in its C_{1b} domain and disables Ca^{2+} /calmodulin activation by interfering with the calmodulin binding site (51).

Conclusions and Perspectives

A great deal of regulatory complexity has been layered onto the rather simple core structure of adenylyl cyclase. The core consists of two parts. The all important two metal ions bind to one part, C_1 . The nucleotide binding pocket and other catalytic residues are contributed primarily by the other part, C_2 . Both parts need to be aligned to carry out catalysis. The most potent activators of the broad range of mammalian adenylyl cyclases, $G_s\alpha$ and forskolin, bind to the domain interface and thereby control domain orientation in a powerful and direct manner. The small molecule forskolin binds on the inside, whereas the large protein activator binds to the outside of the wreath. More specialized regulatory sites have been added to the surface of the catalytic domain ($G\beta\gamma$, protein kinase C) or appended to it (Ca^{2+} /calmodulin, protein kinase A). The structural work reinforces the concept of mammalian adenylyl cyclases as sophisticated coincidence detectors and provides a new framework for a precise understanding of regulation.

Progress in understanding the structure and function of the C_{1a} and C_2 regions has not been matched by information on the rest of adenylyl cyclase. Proposed roles for the transmembrane segments M_1 and M_2 as a transporter or ion channel (8), membrane potential sensor (52, 53), or Ca^{2+} channel interaction domain (54) have yet to be proved or conclusively disproved.

Resolution of this mystery would be a major advance. There are no structural data for C_{1b}, M₁, or M₂. For now we must live with a *Who Framed Roger Rabbit* picture in which we see the catalytic core in vivid three-dimensional "live action" whereas the remainder is just a cartoon.

What has the recent burst of progress in the structure and biochemistry of adenylyl cyclase contributed to the physiology of cAMP signaling? The possibility has been raised that there are endogenous forskolin-like or P-site inhibitor small molecule regulators. The locations of most key regulatory sites are now known. In many cases we can selectively alter the specificity of these sites, eliminate them, or even create them at will by site-directed mutagenesis. Transfection experiments with engineered cyclase isoforms should provide powerful new tools to determine *in vivo* how a particular regulator controls a particular adenylyl cyclase isoform in the context of many simultaneously active pathways.

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